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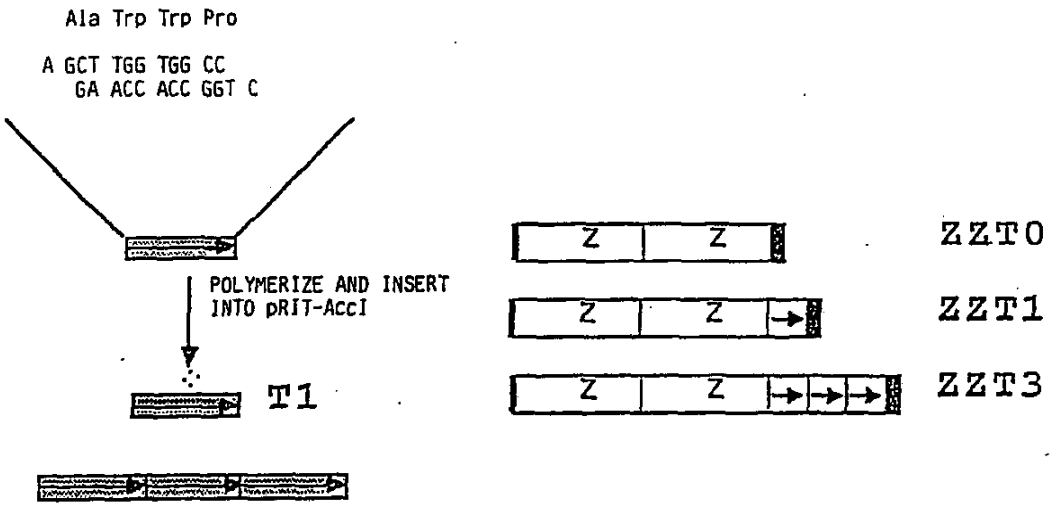

(57) Abstract

A method for isolating and purifying a product peptide or protein originating from cultivation of recombinant cells producing such peptide or protein using an aqueous two-phase system with an upper phase and a lower phase, comprising the steps: a) providing recombinant cells capable of producing a modified product peptide or protein containing tryptophan entities in addition to any ones naturally occuring in said product peptide or protein; b) cultivating the cells resulting from step a) under conditions resulting in expression of said peptide or protein; c) transferring the entire culture, the growth medium or harvested and disrupted cells into an aqueous two-phase system; d) allowing said system to reach an equilibrium resulting in an enrichment of said modified peptide or protein in said upper phase at an increased partition coefficient higher than that obtained with an unmodified peptide or protein; and e) recovering said modified peptide or protein from said upper phase; and peptides and proteins obtained by such method.

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A method for isolating and purifying peptides and proteins.

The invention relates to a general method to enhance the partition coefficient in aqueous two-phase systems of recombinant protein products. In the invented method, the product is modified by the addition of tryptophan entities at the DNA level.

FIELD OF THE INVENTION

The invention relates to methods for the purification of recombinant proteins by aqueous two-phase systems.

BACKGROUND OF THE INVENTION

The use of recombinant DNA techniques for the expression of heterologous proteins has opened new possibilities to produce protein products in quantities. By these methods, the gene encoding the product of interest is introduced into a host cell, eg. bacteria, fungi, yeast or mammalian cells, which can be grown in culture in a way so that the gene will become expressed in the cell. Expressed proteins and peptides can be purified and be used for a number of applications, eg. for pharmaceutical and veterinarian use and to express enzymes of industrial interest eg. for the food industry or to be used as detergents.

A technical problem associated with the use of recombinant protein expression methods, is to recover the protein product free from host components such as cells, cell debris, nucleic acids and host proteins. The level of purity needed is dependent on the specific application; for products to be used as pharmaceuticals, the purity must be very high, typically ≥99%, while the purity for a protein to be used in an industrial application could be lower. Proteins are normally purified by one or more chromatographic methods such as affinity chromatography (AC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), gel filtration chromatography (GFC)

and high pressure liquid chromatography (HPLC). The number of chromatographic steps applied in a purification scheme is approximately proportional to the final level of purity. Thus, pharmaceutical products are often passed through a larger number of chromatographic steps than industrial enzymes.

Before applying a crude extract containing a protein product of interest onto a chromatography column, the protein extract containing the product must be separated from solids (cells and cell debris and nucleic acids). The reason is that all components applied to the column must be able to pass through the gel matrix. Otherwise, the solid and viscous components would compress the gel and eventually stop the liquid flow completely. Thus, a purification step must be included in the process scheme to separate the product from solid and viscous components, such as cells, cell debris and nucleic acids. The most commonly used methods for this purpose are centrifugal separation and/or microfiltration methods. The utilized methology is dependent on the product, host cell type and localization of the product (extracellular, intracellular, bacterial periplasm, etc.). An alternative method as the primary step in a purification scheme is partitioning in aqueous two-phase systems. These methods have been developed during the last 15 years (Sutherland, I.A. and Fischer, D. (1985) in Partitioning in aqueous two-phase systems (Walter, H., Brooks, D.E and Fischer, D., eds.) Academic Press, London, pp627). A two-phase system in water can be formed by a polymer, typically poly(ethylene glycol) (PEG), and a salt. The main benefits of the partitioning technique are the following: The method is (i) efficient, (ii) easy to scale up, (iii) fast when used with continuous centrifugal separators, (iv) relatively low in costs and (v) high in water content which provides for high biocompatibility. Even though considerable savings can be made in large scale, industrial large-scale applications of aqueous two-phase systems to purify proteins are to

date very few. One reason for this is that the partitioning coefficient (K=concentration in top phase/concentration in bottom phase) cannot be accurately predicted.

When a protein is to be isolated from a crude extract by aqueous two-phase partitioning, the more extreme its distribution between the phases is, the more powerful the process step will be. A high partition coefficient provides selectivity and purification relative to the rest of the cell protein. It is further possible to isolate the product using an extreme phase volume ratio, with a volume concentration as the result, and still retaining a high yield. When the partition coefficient is high, as is the case for the intracellular enzyme β-galactosidase, the aqueous two-phase partitioning will provide for a purification and concentration of the product, in addition to the removal of cell particles and nucleic acids in one step. Thus, it is possible to collect the product in a small PEG-rich top phase, and at the same time displacing cell particles and nucleic acids into the salt-rich bottom phase. The search for higher partition coefficients, which provide a possibility to achieve a concentrated product with the yield retained at a high level, has lead to the construction of a number of "second-generation" aqueous two-phase systems. One example is affinity partitioning, where PEG with covalently coupled affinity groups are used as the polymer component to make the product of interest to bind to the modified PEG. These, and other similar approaches, can make aqueous two-phase extraction very selective for essentially any product. However, the high cost of the modified PEG, problems to find a suitable affinity group and the necessity to recycle the modified PEG for economical reasons, make this concept unattractive in large scale situations. Therefore, if recombinant proteins could be engineered to partition to the top phase in an aqueous two-phase system in a predicted manner, this would open new possibilities in utilizing aqueous two-phase ex-

traction technology as a general first step in the recovery of protein products.

Recombinant DNA techniques have been used to engineer proteins to facilitate their purification as a fusion protein. In a gene fusion system, a gene fragment encoding the product protein can be fused to a gene encoding a property which can be utilized to simplify the purification. The most common property of the fusion partner is to have a strong affinity to a ligand which will be utilized to purify the expressed recombinant fusion protein by ligand affinity chromatography. To date, more than 10 different such affinity handle systems have been developed. One disadvantage by these methods, is that chromatography must be preceeded by a purification step to remove solids and nucleic acids, which could cause problems, as the affinity chromatography step preferentially should be applied as early as possible in the process. Especially for the isolation of intracellular recombinant products, sophisticated separation steps will be needed in the process scheme after cell disruption, before loading the crude extract onto the affinity column. In addition, chromatography techniques are often limited by their poor mass-transfer rates and the chromatography resins are often expensive, which will make scaling-up an economical problem.

The described invention is a novel gene fusion approach generating enhanced partitioning properties in aqueous two-phase systems of a recombinant protein as a fusion protein. One way to achieve high partition coefficients would be to fuse the product of interest to a protein which shows high partition coefficient, thereby hoping to accomplish extreme partition properties also when fused to protein products. This concept has two major disadvantages: (i) The system would not be flexible: If the fused peptide would lower the partition coefficient too much by the properties of the product, the concept could not be used. (ii) Gene products with extreme partitioning properties are typically very large, eg. E.coli β-

galactosidase is a tetramer of a monomer consisting of more than 1000 amino acid residues. Thus, by making a fusion to β-galactosidase of a peptide hormone, which typically are of smaller sizes than 100 amino acid residues, the product peptide would account for less than 10% of the fusion protein which will lower the yield of the peptide hormone.

It has now been unexpectedly found that substantially enhanced partition coefficients are obtained by introducing into a peptide or protein of interest tryptophan entities. An example of introduction of tryptophan residues to a protein structure is exemplified by making a fusion to a peptide sequence containing tryptophan residues. Such a peptide is designated a partitioning peptide.

Summary of the invention

The present invention provides for a method to enhance or increase the partition coefficient of recombinant proteins or peptides in favour of the top-phase in aqueous two-phase systems by modifying the product peptide or protein by introducing tryptophan entities into the polypeptide chain of the product. The modification is performed at the DNA level.

The invention relates to a method for isolating and purifying a product peptide or protein originating from cultivation of recombinant cells, and the method is based on the use of an aqueous two-phase system with an upper phase and a lower phase. More specifically, the method of the invention comprises the following steps:

a) providing recombinant cells capable of producing a modified product peptide or protein containing tryptophan entities in addition to any ones naturally occurring in said product peptide or protein;

b) cultivating the cells resulting from step a) under conditions resulting in expression of said peptide or protein;

c) transferring the entire culture, the growth medium or harvested and disrupted cells into an aqueous two-phase system;

d) allowing said system to reach an equilibrium resulting in an enrichment of said modified peptide or protein in said upper phase at an increased partition coefficient higher than that obtained with an unmodified peptide or protein; and

e) recovering said modified peptide or protein from said upper phase.

According to a preferred aspect of the invention such modified peptide or protein comprises a peptide or protein obtained by fusion at the DNA-level of a gene encoding said product peptide or protein and another gene encoding a fusion partner containing tryptophan entities. If desired, said fusion partner can be removed from the modified peptide or protein recovered from said upper phase.

According to a preferred embodiment of the invention said fusion partner in the modified peptide or protein contains a tryptophan dipeptide sequence. More particularly, said fusion partner may contain the amino acid sequence:

$Ala(Trp)_nPro$, where n is an integer ≥ 2.

To further enhance or increase the partition coefficient the fusion partner may contain the following amino acid sequence:

$[Ala(Trp)_nPro]_m$, where n has the above meaning and m is an integer ≥ 1.

In said sequence m may be an integer from 1 to about 15.

As will be clear from the following exemplification of preferred embodiments said fusion partner may additionally contain a derivative of the Staphylococcus protein A. It is particularly preferred that said derivative is capable of binding to the Fc region of human immunoglobulin G.

Although introduced tryptophan entities may be fused into the polypeptide chain of the product peptide or protein at any suitable site thereof it is preferred that said fusion partner is fused to the C-terminus or N-terminus of the product peptide or protein.

Product proteins of interest may include but are not limited to proteins of pharmaceutical interest such as polypeptide hormones, eg. insulinlike growth factor 1 (IGF-1), IGF-2, growth hormone (GH), parathyriod hormone (PTH), gonadotropins (folicle stimulating hormone (FSH), proluteinizing hormone, chorionogonadotropin), insulin, prolactin, placental lactogen, relaxin and thyrotropin. Other polypeptides of pharmaceutical interest may also be included, eg. calcitonin, enkephalin, cytokines, α-interferons, β-interferons, γ-interferons, blood coagulation factors (eg. Factor VIII) and thrombolytic proteins (eg. tissue plasminogen activator and streptokinase). Such products may also include proteins or polypeptides to be used in industrial or dairy applications, eg. enzymes such as proteases (eg. subtilisin and rennin), lipases, amylases, cellulases and ligninases. Such product peptides and proteins may be of prokaryotic, eukaryotic as well as of synthetic origin.

The designed partitioning peptide is described in Example 1. The sequence was designed to $(AlaTrpTrpPro)_n$. A synthetic gene encoding the peptide sequence was designed to be possible to polymerize in an obligate head-to-tail fashion so that the number of fused peptide sequences may be varied to essentially any multiplicity. A gene encoding the peptide sequence was synthesized, cloned and analyzed, and gene fragments encoding 1 and 3 (n=1 and 3, respectively) partitioning peptides were fused to a model protein. As a model protein to study the effect of the partitioning peptide, a derivative of staphylococcal protein A (ZZTO) was chosen. The even distribution of ZZTO in the PEG4000/potassium phosphate system makes it suitable for the analysis. In addition, the IgG-binding property pro-

vides for easy purification. The ZZT0 protein and the derivatives with 1 and 3 fused partitioning peptides, designated ZZT1 and ZZT3, respectively, were expressed and purified by IgG affinity chromatography (Example 4). The partitioning coefficients of the fusion proteins ZZT0, ZZT1 and ZZT3 were analyzed and the ZZT1 and ZZT3 showed dramatically increased partitioning to the top phase in the three tested PEG4000/potassium aqueous two-phase systems. The system with the highest value of ΔC_{PEG} shows the highest K values and the largest differences between the proteins. In this extraction system, the addition of one partitioning peptide to the ZZT0 protein, which has a size of 147 amino acids, makes the K value increase more than 7 times. By fusion of the trimer of the partitioning peptide to ZZT0, the K value was determined to increase 60 times. The true increase may be even higher as virtually all full-length protein was found in the top phase, while the protein in the bottom phase was mainly degradation products.

In spite of the presence of the partitioning peptide, the expression levels were high for all threee proteins: 13, 33, 7 mg of product/g dry cells for ZZT0, ZZT1 and ZZT3, respectively. These expression levels correspond to about 1.3%, 3.3% and 0.7%, respectively, of total cell weight being the product protein.

Although this invention is not limited to a specific theory, an explanation for the dramatic effects on the partition coefficient by fused tryptophans might be found in a specific interaction between tryptophan and the polymer component of the upper phase, such as PEG. Tryptophan, with its indole ring, is the only natural amino acid capable of entering into charge-transfer interaction as a donor. The pyrrole nitrogen of the indole ring may also be a hydrogen donor in hydrogen bond interaction. An important feature of the molecular structure of PEG, providing for its water solubility, is the repeating ether oxygen linkage with lone electrone pair making this a powerful

hydrogen bond acceptor. In the absence of hydrogen donating groups, to balance the hydrogen bond accepting capacity in the molecular structure, PEG has been characterized as a monopolar Lewis base.

EXAMPLES

The following examples are provided to illustrate the invention, and are not to be construed as limiting the scope of the invention.

EXAMPLE 1

Design of a partitioning peptide

A partitioning peptide sequence was invented, which should enhance the partition coefficient of the product as a fusion protein in an aqueous two-phase system. In addition, the gene fragment encoding the peptide should be possible to polymerize at the DNA level to any multiplicity in an obligate head-to-tail fashion, so that the system can be optimized for each product by varying the multiplicity of the peptide. We decided to design a peptide sequence, that contained surface exposed tryptophan residues. As tryptophan is considered a hydrophobic amino acid residue, there is an obvious risk that fused tryptophans will hide from the solvent by folding back into the protein interior, form a hydrophobic pocket or even disrupt the folding of the attached product by hydrophobic effects. To minimize this risk, a peptide with minimal conformational freedom was invented. First, frequent proline residues were decided to be used in the peptide, as the peptide bond in prolines shows restrictions. Secondly, tryptophans were designed as neighbouring amino acids as the bulky side-chains of the tryptophans will result in sterical hindrance for the polypeptide backbone to rotate. An alanine residue had to be used as a spacer, but this residue did not introduce too much conformational freedom to the peptide sequence. Thus, the partitioning peptide to be used as an example in the invention, was designed to

$(AlaTrpTrpPro)_n$, where n is an integer ≥1. The peptide sequence was displayed on a computer graphics work station (Personal IRIS from Silicon Graphics) using the commercially available software from Biosym, San Diego, USA. The molecular dynamic behaviour of this peptide sequence as a trimer (n=3) was calculated using DISCOVER with the AMBER force-field (by using the software from Biosym, San Diego, USA). The conformation of the 12 amino acids long polypeptide chain remained as an extended structure through out the calculations (3000 fs). Based on this modelling result, it is likely that the conformational freedom of the designed peptide sequence is restricted. The structures of the AlaTrpTrpPro and $(AlaTrpTrpPro)_3$ peptides are shown as computer graphics representations in Figure 1.

EXAMPLE 2

Design, synthesis, polymerization and cloning of a partitioning peptide gene fragment.

All the recombinant DNA manipulations in Example 2 followed standard procedures (Maniatis, T., Fritsch, E. and Sambrook, J. 1982. Molecular cloning: A laboratory handbook. Cold Spring Harbor Laboratory Press, New York) unless stated others, and enzymes are used according to the recommendations by the suppliers. A gene fragment encoding the designed partitioning peptide was made synthetically by the synthesis of two oligonucleotides, 5'-AGCTTGGTGGCC-3' and 5'-CTGGCCACCAAG-3'. The oligonucleotides were synthesized on a Gene Assembler (Pharmacia, Uppsala, Sweden). The oligonucleotides were purified by standard techniques, kinased and dissolved separately in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). Upon mixing, these oligonucleotides will anneal and form a gene fragment encoding the partitioning peptide and with non-palindromic sticky-ends to facilitate polymerization in an obligate head-to-tail fashion (Figure 2A). The oligonucleotides were mixed in a ligation buffer to a concentration of 10 µg/ml of each oligonucleotide. In addition, the

plasmid vector pRIT-AccI (Ljungquist, C., Breitholtz, A., Brink-Nilsson, H., Moks, T., Uhlén, M. and Nilsson, B. 1989. Eur.J.Biochem. 186:563-569) which had been cleaved with Acc1 was added to the ligation mixture to a concentration of 5 µg/ml. T4 DNA Ligase (New England Biolabs) was added and the reaction mixture was incubated at 4°C for 10 hours. Thereafter, 10 µl of the ligation mixture was transformed into E.coli HB101 (Boyer, H. and Roulland-Dussoix, D. 1969. J.Mol.Biol. 41:459-472). E.coli colonies containing pRIT-Acc1 with inserted fragments of one (designated T1) and three (designated T3) partitioning peptide gene fragments, respectively, were selected for further studies. The nucleotide sequences spanning the T1 and T3 gene fragments, respectively, were determined using dideoxy sequencing and analyzed on an Automated Laser Fluorescence Sequensor (Pharmacia-LKB, Sweden). The plasmid vectors harbouring the cloned partitioning peptide gene fragments were designated pRIT-Acc1-T1 and pRIT-Acc1-T3, respectively.

EXAMPLE 3

Fusing the partitioning peptide gene fragments to a gene encoding two domains of a synthetic IgG-binding domain based on staphylococcal protein A (ZZ).

All the recombinant DNA manipulations in Example 3 followed standard procedures (Maniatis, T., Fritsch, E. and Sambrook, J. 1982. Molecular cloning: A laboratory handbook. Cold Spring Harbor Laboratory Press, New York) unless stated others, and enzymes are used according to the recommendations by the suppliers. The plasmid vector pHAZY:475 (Altman, J.D., Nilsson, B., Andersson, S. and Kuntz, I.D. 1990, submitted to Protein Engineering) used for efficient intracellular expression of Z fusions to bovine pancreatic trypsin inhibitor, was used in this study. This plasmid has the Z gene (Nilsson, B., Moks, T., Jansson, B., Abrahmsén, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T.A. and Uhlén, M. 1987 Protein

Engineering 1, 107-113) under transcriptional control of the E.coli trp promoter (Yansura, D.G. and Henner, D.J. 1990 Methods in Enzymology 185, 54-60). The pHAZY:475 plasmid was digested with ClaI and SalI and the fragment was exchanged to the corresponding ClaI/SalI fragment from pBR322ΔHindIII by ligating the fragments in vitro followed by transformation into E.coli HB101 (Boyer, H. and Roulland-Dussoix, D. 1969. J.Mol.Biol. 41:459-472). The resulting plasmid, pHAZY:475ΔH, is identical to pHAZY:475, but lacks the HindIII restriction site just upstreams of the tetracyklin resistance gene. The plasmid pBR322ΔHindIII was constructed by in vitro mutagenes. In the mutagenesis, the oligonucleotide 5'-AGCTTATCATCGATAAGCTATAATGCGG-3' changed the HindIII recognition sequence AAGCTT to AAGCTA, as defined by the tet gene plus strand in pBR322.

The ZZ domains originate from the plasmid pEZZT308 (Nygren, P-Å., Eliasson, M., Palmcrantz, E., Abrahmsén, L. and Uhlén, M. 1988. J.Mol. Recognition 1:69-74), which was partially digested with BglII and then cleaved with EcoRV. The 615 bp fragment spanning approximately 1.5 Z domains, the mp8 multirestriction enzyme linker and the E.coli trp transcription terminator, was ligated into pHAZY:475ΔH previously digested with BglII and StuI. This creates the gene fusion vector, pRIT44 designed for intracellular expression of ZZ fusions with both ampicillin and tetracyclin resistance. The expression is under control of the E.coli trp promoter and the first 8 amino acids in the fusion protein originate from the Trp leader. The vector pRIT44 was used to produce ZZT0, the control protein in the partitioning experiments. The nucleotide sequence of the ZZT0 gene is shown in Figure 3.

DNA fragments containing 1 or 3 handle sequences were obtained by digesting the pRIT-AccI-T1 and pRIT-AccI-T3 plasmids with EcoRI and HindIII and isolating the fragments on a 3% agarose gel. After electroelution, the fragments were ligated into pRIT44, restricted with EcoRI and

HindIII, creating the plasmids pRIT44T1 and pRIT44T3. Schematic representations of the ZZT0, ZZT1 and ZZT3 proteins are shown in Figure 2B. The nucleotide sequences as well as the amino acid sequences are shown in Figure 3. A plasmid map of pRIT44T3 is shown in Figure 4. E.coli HB101 harbouring pRIT44T1 has been deposited at the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

EXAMPLE 4

Expression and purification of ZZT0, ZZT1 and ZZT3.

The ZZ fusion proteins were expressed in E.coli RV308 (Maurer, R., Meyer, B.J. and Ptashne, M. 1980. J.Mol.Biol. 139:147-161). Cultures harbouring each different plasmid, pRIT44T0, pRIT44T1 and pRIT44T3, respectively, were grown in a 2L scale in a bioreactor (Belach 3 L bioreactor, type FLC-3-A, Belach, Sweden). The cultivation medium consisted of (in g L^{-1}):KH_2PO_4, 2; K_2HPO_4, 3; $(NH_4)_2SO_4$, 2.5; Na_3-citrate, 0.5; yeast extract, 2; casamino acids, 20; glucose, 20; ampicillin, 0.1; tetracyclin, 0.008; thiamine, 0.07. Also, 2 mL L^{-1} of 1 M $MgSO_4$ and 2 ml of a trace element solution. pH was controlled at 7.0 with 25% NH_3. Cell growth was monitored by following the optical density (OD) at 580 nm. Temperature was kept at 37°C during the first part of the cultivation. Transcription from the trp promoter was induced by adding 25 mg/L indole acrylic acid (Sigma), at OD 18, 4.5 and 1.7 for ZZT0, ZZT1 and ZZT3, respectively. At induction, the cultivation temperatures were lowered to 30°C. Cells were harvested after 3 hours of induction by centrifugation (20 min, 3300 x g), resuspended to 50% wet weight in TS buffer (25 mM Tris-HCl,pH 7.4, 200 mM NaCl) and stored in -80°C until further use.

Cells were thawed and disintegrated in a high pressure homogenizer (French press FA-073, Aminco, USA). The disintegrate was diluted to 25% by addition of TST buffer (TS buffer containing 0.05% Tween 20). In the ZZT0 preparation, centrifugation of the disintegrate at 25000 x g

for 20 min followed. The supernatant was then furnished with 0.5 mL cold saturated $(NH_4)_2SO_4$ per mL and kept stirred on ice for 15 min. A second centrifugation was performed at 35000 x g for 20 min. In the perparations of ZZT1 and ZZT3, the precipitation step was omitted. Instead, the disintegrate was centrifuged at 35000 x g for 20 min. The supernatant was collected and centrifuged. The proteins were further purified with affinity chromatography using IgG-Sepharose Fast Flow (Pharmacia, Sweden). The column was equilibrated with TST buffer and loaded with the supernatant from the second centrifugation, washed with TST, and equilibrated with 5 mM NH_4Ac. The ZZ proteins were eluted with 200 mM HAc titrated to pH 3.3. The eluted fractions were lyophilized and stored at -20°C.

The yields of ZZ proteins were approximately 160 mg ZZT0, 220 mg ZZT1 and 15 mg ZZT3/liter culture, respectively. This corresponded to, in mg protein/g dry weight of cells 13, 33 and 7, respectively. The IgG purified proteins were analyzed by SDS-PAGE (Figure 5 lanes 2, 5 and 8). In addition to proteins corresponding in size to the molecular weight of ZZ, which are the approximate molecular weights of ZZT0, ZZT1 and ZZT3, there are bands of smaller sizes, most notably for ZZT1 and ZZT3. These bands are most likely products derived from proteolytic degradation of the full-length protein in vivo.

EXAMPLE 5

Partitioning of ZZT0, ZZT1 and ZZT3 in PEG 4000/potassium phosphate aqueous two-phase systems.

The partition coefficients of the proteins ZZT0, ZZT1 and ZZT3 in phase systems consisting of PEG4000/potassium phosphate have been investigated. The partitioning experiments were performed with the IgG affinity purified preparations of the proteins. Each protein was dissolved in distilled water. Phase systems were prepared from 40% (w/w) stock solutions of PEG4000 and potassium phosphate (pH7) in graded centrifugal tubes. Three different phase

system compositions were used, representing increasing ΔC_{PEG}. The phase systems were chosen as to obtain a top to bottom phase volume ratio of 1:1, in order to provide more accurate sampling procedures. Appropriate amounts of potassium phosphate, PEG4000 and distilled water were weighed into a tube (total weight of each phase system was 5 g). The content was thoroughly mixed before the concentrated protein solution was added. Approximately 0.7 OD units were added to each phase system. After mixing, the phase systems were incubated at 20°C for 5 minutes, centrifuged at 500 x g for 4 minutes and incubated at 20°C for a final 5 minutes. Samples from top and bottom phases were carefully withdrawn with pasteur pipettes. When partitioning ZZT3 for the study of degradation product distribution, a 10 g phase system with approximately the same protein concentration as in the previous ZZT3 experiments was used. The larger phase system was needed in order to obtain sufficient amounts of ZZT3 for the SDS-PAGE analysis of the bottom phase. Partition coefficients, defined as the ratio of protein concentrations in top and bottom phases, were measured spectrophotometrically. The absorbances at 280 and 310 nm were used. Each top and bottom phase sample was referenced against an identical, but protein free top or bottom phase, respectively. The partition coefficient increased dramatically for the ZZ proteins with fused partitioning peptides (Table 1, Figure 6). Also, the increase in partition coefficient that is possible to obtain by altering the phase system composition (i.e. altering ΔC_{PEG}) was magnified several times, when the number of peptide handels increased. These results clearly demonstrate that the designed partitioning peptide had a large impact on the partition coefficients. Further, the proteins in the top and bottom phases were analyzed by SDS-PAGE (Figure 5). SDS-PAGE was performed using standard procedures (Laemmli, U. 1970. Nature 227:680-685) with 3.5% stacking gel and 15% separation gel. For evaluation of expression and degradation patterns during cultivation,

IgG-purified proteins were used. Top and bottom phase samples were applied to IgG-Sepharose, eluted by 200 mM HAc and lyophilized. Each preparation was thereafter dissolved in distilled water and equal amounts of material were loaded onto SDS-PAGE. Equal amounts of protein were loaded into each lane, which means that the relative amounts in the top and bottom phases for each protein are not reflected in this electrophoretic analysis. Interestingly, the degradation products of ZZT1 and ZZT3 are predominantly distributed to the bottom phases (Figure 5, lanes 6 and 9). Thus, the presence of the partitioning peptides in the full length materials resulted in increased partitioning to the top phase relative to the degradation products. To verify increasing amounts of tryptophans in the purified ZZ fusion proteins, the UV spectra of the proteins from the top phases were analyzed. The UV spectra were determined using a Kontron 860 spectrophotometer. Samples were prepared from top-phase materials, and diluted in water to an OD at 220 nm of 9. The same amounts of proteins (absorbance at 220 nm) were analyzed. The results show that the absorbance at 280 nm increases stepwise with the expected increase in tryptophan content (Figure 7). As tryptophan shows a strong absorbance at 280 nm, these results are consistent with the presence of the partitioning peptides in the ZZT1 and ZZT3 fusion proteins.

	ZZT0	ZZT1	ZZT3
Mw(kDa)	16.6	17.1	18.2
Expression level	13	33	7
Number of Trp	0	2	6
$K_{(10.1/9.7)}$	0.9	1.6	2.7*
$K_{(10.4/12.8)}$	1.4	3.6	21
$K_{(10.8/13.3)}$	1.6	12	96

Table 1
Different charactersistics of the recombinant proteins ZZT0, ZZT1 and ZZT3. The expression level refers to the fermentor cultivations as described in the experimental protocol. The levels are given in mg fusion protein/g dry cell weight. The partition coefficient, K, for the three recombinant proteins are given for three different compositions of the PEG4000/potassium phosphate system. The composition is given as (% salt/%PEG).
* = phase system composition was 10.2/9.8.

FIGURES

Figure 1
Computer graphics representation of the AlaTrpTrpPro and the $(AlaTrpTrpPro)_3$ peptides.

Figure 2
A; The nucleotide sequence and the polymerization procedure of the gene fragment encoding AlaTrpTrpPro. B; Schematic representation of the recombinant proteins used in this stude. Z represents the synthetic IgG-binding domain based on domain B from SPA and the arrows represent the partitioning peptide polymerized in a head-to-tail fashion.

Figure 3
Nucleotide sequence of the gene encoding the ZZTO protein, including 19 nucleotides 5' of the ATG start codon and 27 nucleotides 3' of the TAA stop codon. The putative amino acid sequence of the ZZTO protein is shown. In addition the DNA and amino acid sequences of the T1 and T3 fragments are shown. Arrow shows the position in ZZTO where the T1 and T3 sequences are inserted, respectively.

Figure 4
Plasmid map of pRIT44T3 encoding the ZZT3 fusion protein. TET is the tetracyklin resistance gene from pBR322, AMP in the β-lactamase gene from pBR322, Ptrp is the trp promoter and ZZT3 shows the gene encoding ZZT3. The three alligned filled arrows represent the three partitioning peptide gene fragments. Some restriction sites are shown and the two restriction sites shown in bold show the sites used to clone the partitioning peptides from pRIT-AccI-T3 into pRIT44.

Figure 5
Analysis of the distribution of full-length protein and

degradation proteins between the top and bottom phases in aqueous two-phase partitioning by 15% SDS-PAGE. Lanes 1 and 11 are marker proteins with sizes shown as mol. mass x 10^{-3}. Lanes 2, 3 and 4 are starting material, top and bottom phases, respectively, of ZZT0; lanes 5, 6 and 7 correspond to ZZT1 samples in the same order and lanes 8, 9 and 10 to ZZT3.

Figure 6

The distribution of the fusion proteins in a PEG4000/potassium phosphate aqueous two-phase system (pH 7, 20°C) is shown as the partition coefficient (K), in the ordinata, at different ΔC_{PEG} (i.e. the difference between PEG concentration in the top and bottom phases), in the abscissa. Symbols: ZZT0, ZZT1 and ZZT3.

Figure 7

UV absorbance spectra of ZZT0, ZZT1 and ZZT3 from 240 to 350 nm. The lower spectrum refers to ZZT0, the middle to ZZT1 and the top spectrum to ZZT3. The samples were diluted to have the same absorbance (of about 9) at 200 nm.

International Application No PCT/SE91/00732

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 12, lines 20-22 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution (including postal code and country) [5]

Mascheroder Weg 1 B
D-3300 BRAUNSCHWEIG, Germany

Date of deposit [6]	Accession Number [7]
November 6, 1990	DSM 6227

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Jürgen Wilken
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS

1. A method for isolating and purifying a product peptide or protein originating from cultivation of recombinant cells producing such peptide or protein using an aqueous two-phase system with an upper phase and a lower phase, comprising the steps:

a) providing recombinant cells capable of producing a modified product peptide or protein containing tryptophan entities in addition to any ones naturally occurring in said product peptide or protein;

b) cultivating the cells resulting from step a) under conditions resulting in expression of said peptide or protein;

c) transferring the entire culture, the growth medium or harvested and disrupted cells into an aqueous two-phase system;

d) allowing said system to reach an equilibrium resulting in an enrichment of said modified peptide or protein in said upper phase at an increased partition coefficient higher than that obtained with an unmodified peptide or protein; and

e) recovering said modified peptide or protein from said upper phase.

2. A method according to claim 1, wherein said modified peptide or protein is constituted by a peptide or protein obtained by fusion at the DNA level of a gene encoding said product peptide or protein and another gene encoding a fusion partner containing tryptophan entities.

3. A method according to claim 2, comprising the further step of removing said fusion partner from the modified peptide or protein recovered in step e).

4. A method according to claim 2 or 3, wherein said fusion partner in said modified peptide or protein contains a tryptophan dipeptide sequence.

5. A method according to claim 4, wherein said fusion partner contains the amino acid sequence:

$Ala(Trp)_nPro$, where n is an integer ≥ 2.

6. A method according to claim 5, wherein said fusion partner contains the amino acid sequence:

$[Ala(Trp)_nPro]_m$, where n has the above meaning and m is an integer ≥1.

7. A method according to claim 6, wherein said fusion partner contains the amino acid sequence:

$[Ala(Trp)_nPro]_m$, where n is 2 and m is an integer from 1 to 15.

8. A method according to any of claims 2 to 7, wherein said fusion partner further contains a derivative of the Staphylococcus protein A.

9. A method according to claim 8, wherein said derivative is capable of binding to the Fc region of human immunoglobulin G.

10. A method according to any of claims 2 to 9, wherein said fusion partner is fused to the C-terminus or N-terminus of said product peptide or protein.

11. The product peptide or protein whenever obtained by the method of any preceding claim.

FIG.1

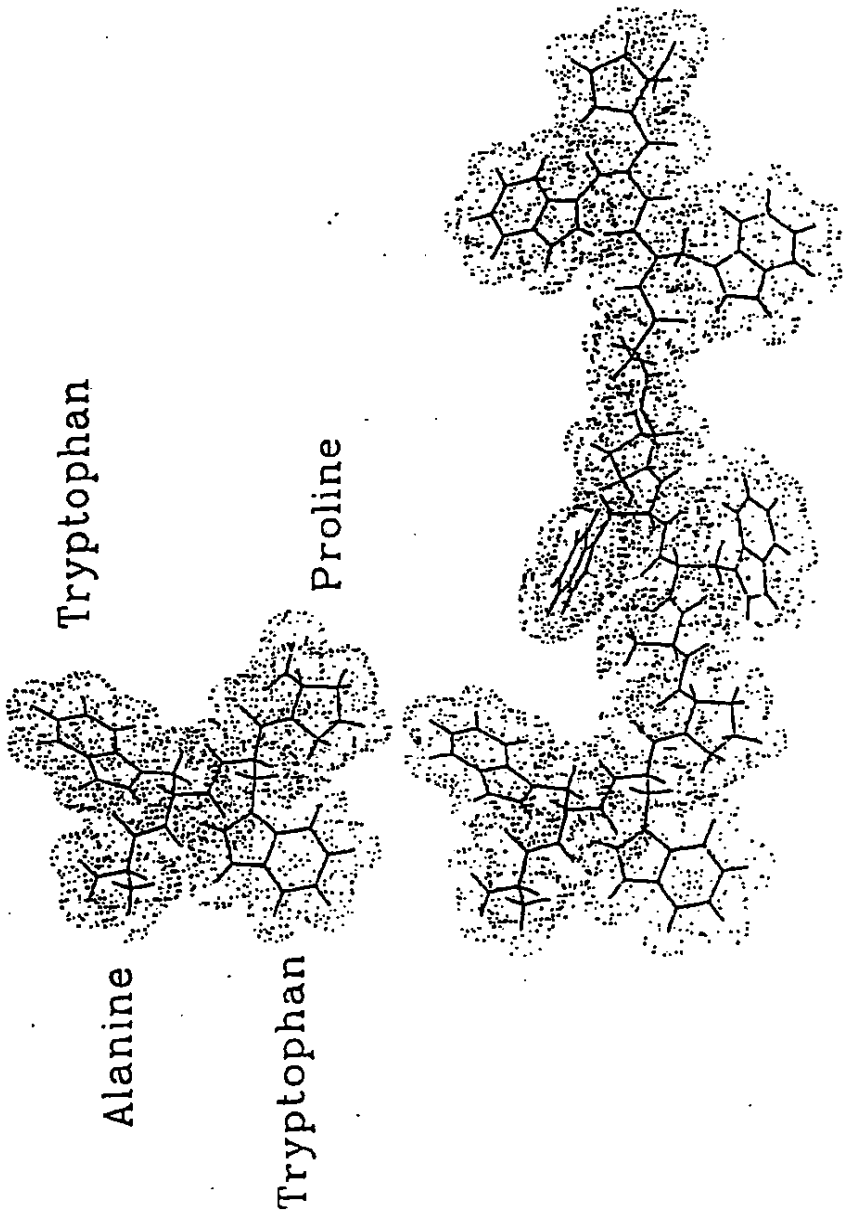


FIG. 2A

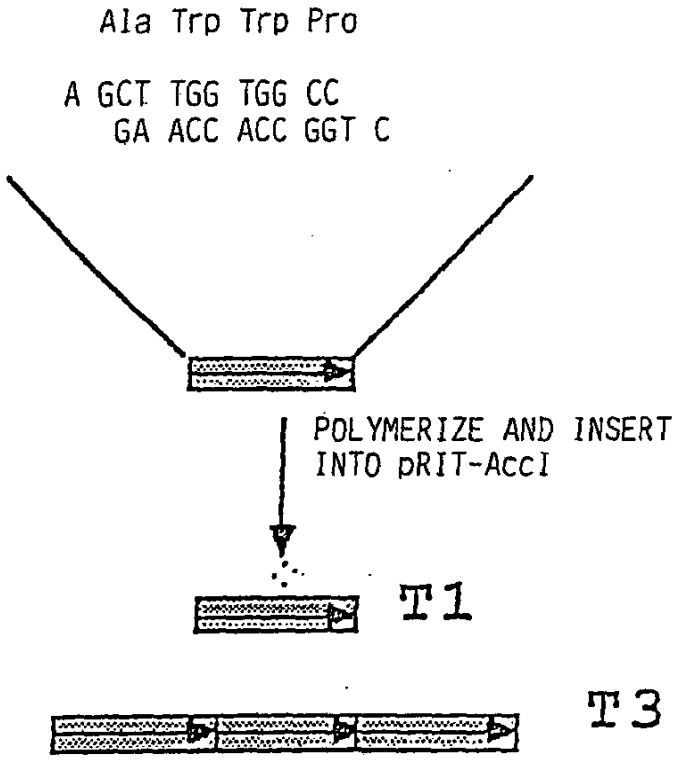
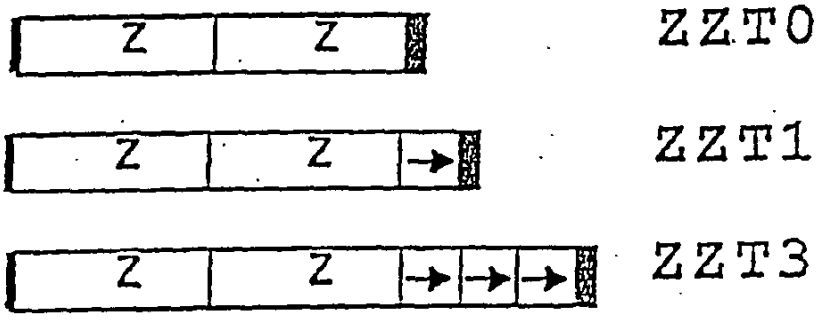
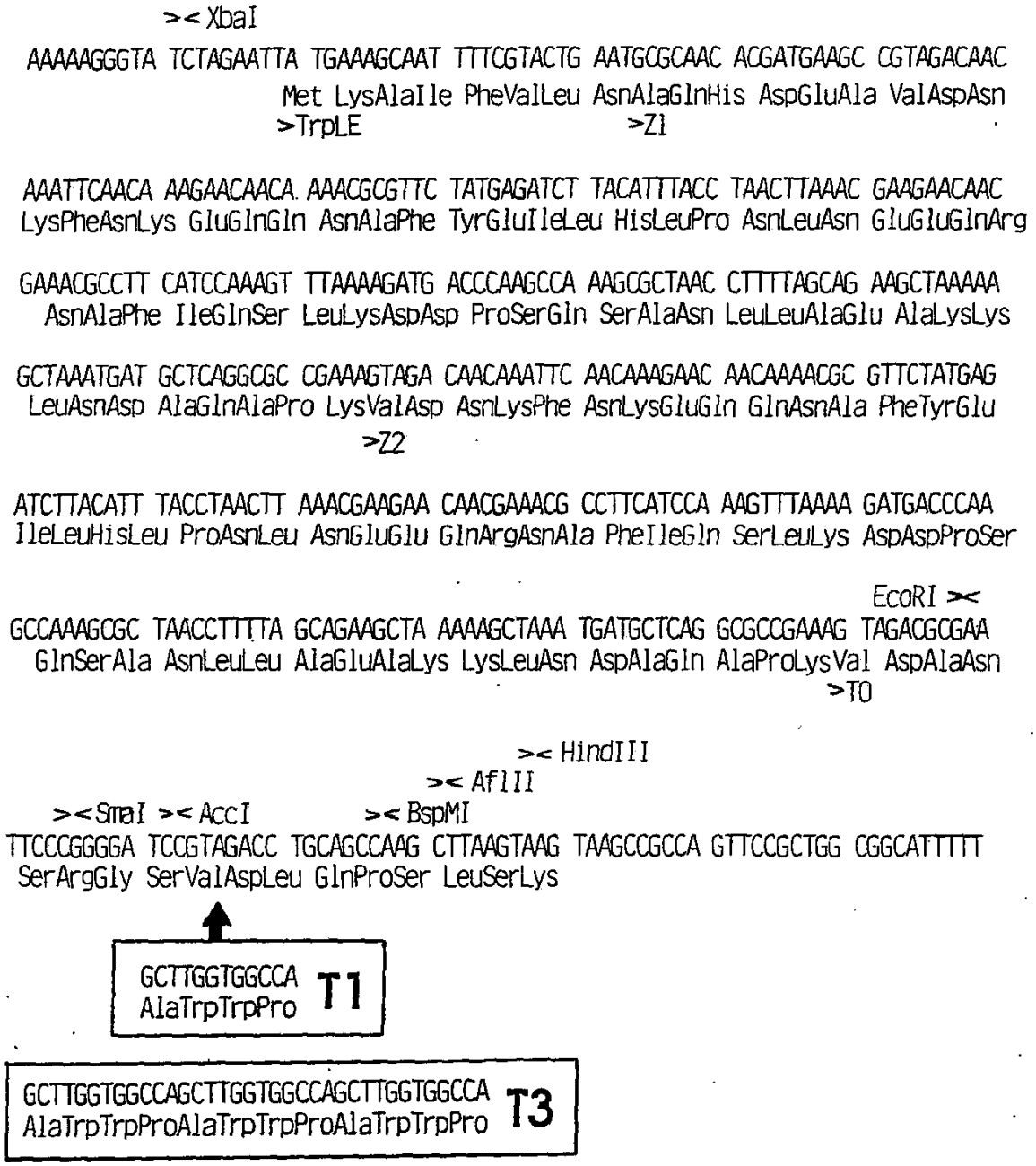

Ala Trp Trp Pro
A GCT TGG TGG CC
GA ACC ACC GGT C
POLYMERIZE AND INSERT
INTO pRIT-AccI
T1
T3

FIG. 2B


Z
Z
ZZT0
ZZT1
ZZT3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>TrpLE
>Z1
AAATTCAACA AAGAACAACA AAACGCGTTC TATGAGATCT TACATTTACC TAACTTAAAC GAAGAACAAC
LysPheAsnLys GluGlnGln AsnAlaPhe TyrGluIleLeu HisLeuPro AsnLeuAsn GluGluGlnArg
GAAACGCCTT CATCCAAAGT TTAAAAGATG ACCCAAGCCA AAGCGCTAAC CTTTTAGCAG AAGCTAAAAA
AsnAlaPhe IleGlnSer LeuLysAspAsp ProSerGln SerAlaAsn LeuLeuAlaGlu AlaLysLys
GCTAAATGAT GCTCAGGCGC CGAAAGTAGA CAACAAATTC AACAAAGAAC AACAAAACGC GTTCTATGAG
LeuAsnAsp AlaGlnAlaPro LysValAsp AsnLysPhe AsnLysGluGln GlnAsnAla PheTyrGlu
>Z2
ATCTTACATT TACCTAACTT AAACGAAGAA CAACGAAACG CCTTCATCCA AAGTTTAAAA GATGACCCAA
IleLeuHisLeu ProAsnLeu AsnGluGlu GlnArgAsnAla PheIleGln SerLeuLys AspAspProSer
EcoRI ><
GCCAAAGCGC TAACCTTTTA GCAGAAGCTA AAAAGCTAAA TGATGCTCAG GCGCCGAAAG TAGACGCGAA
GlnSerAla AsnLeuLeu AlaGluAlaLys LysLeuAsn AspAlaGln AlaProLysVal AspAlaAsn
>T0
>< HindIII
>< AflII
>< SmaI >< AccI >< BspMI
TTCCCGGGGA TCCGTAGACC TGCAGCCAAG CTTAAGTAAG TAAGCCGCCA GTTCCGCTGG CGGCATTTTT
SerArgGly SerValAspLeu GlnProSer LeuSerLys
GCTTGGTGGCCA
AlaTrpTrpPro T1
GCTTGGTGGCCAGCTTGGTGGCCAGCTTGGTGGCCA
AlaTrpTrpProAlaTrpTrpProAlaTrpTrpPro T3

FIG.3

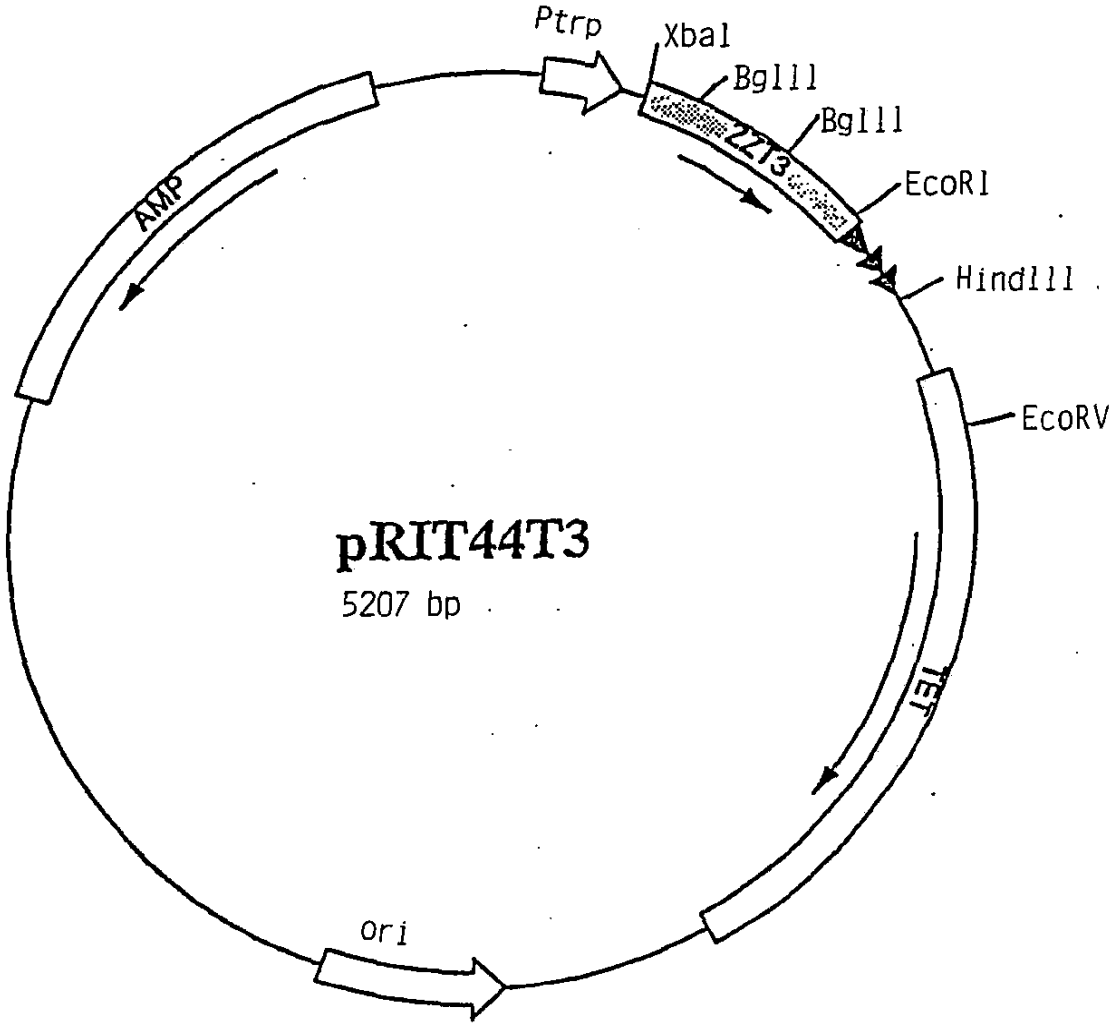

Ptrp
XbaI
BglII
BglII
ZZT3
EcoRI
HindIII
EcoRV
AMP
TET
ori
pRIT44T3
5207 bp

FIG.4

FIG.5

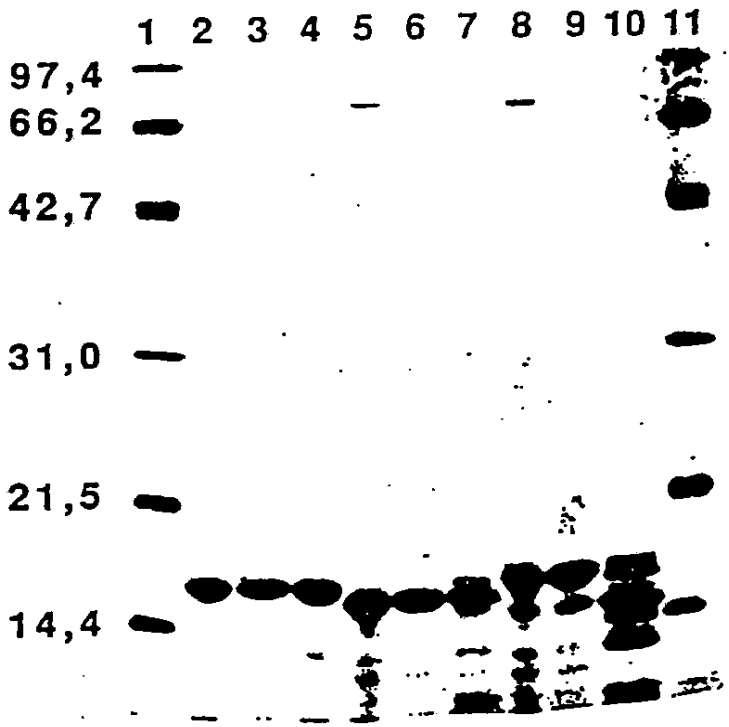
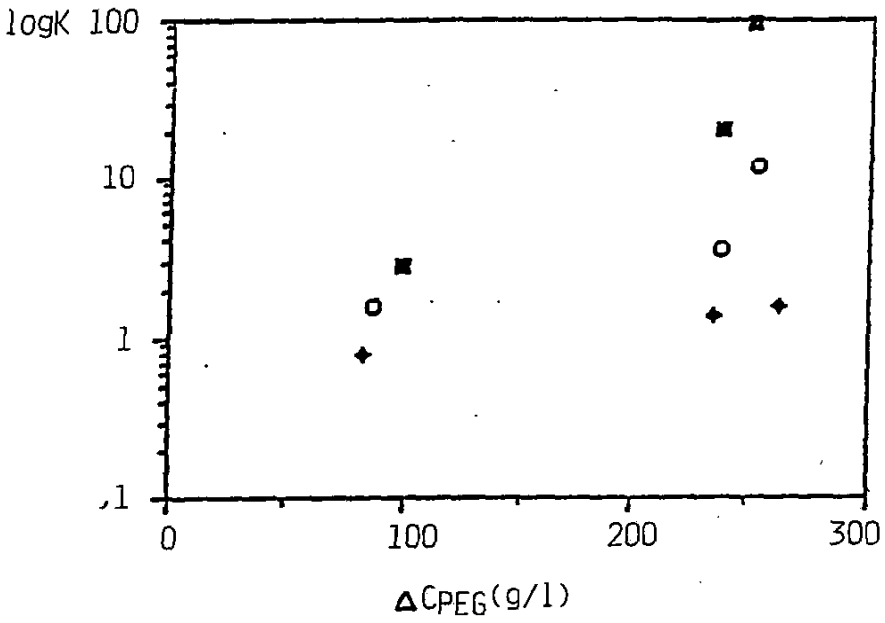


FIG. 6



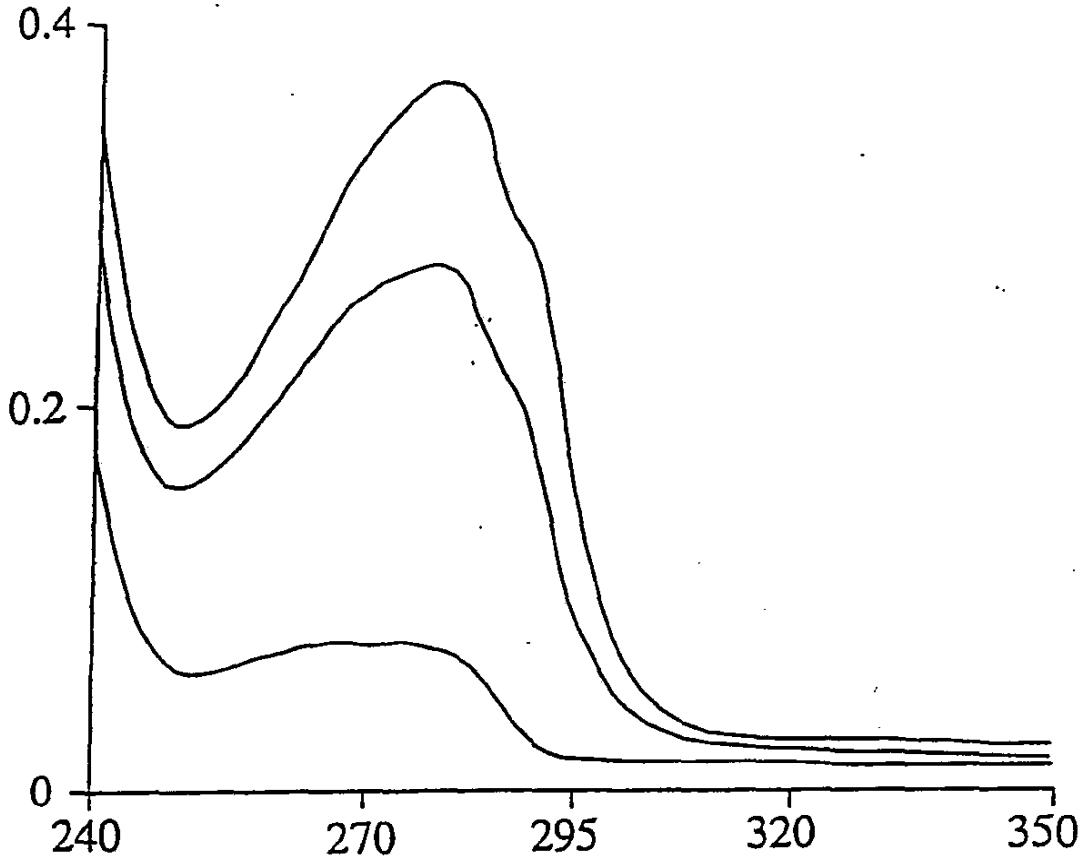


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00732

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 3/12, C 12 N 15/62, C 07 K 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	EP, A1, 0387760 (FORSCHUNGSZENTRUM JÜLICH GMBH) 19 September 1990, *see the whole document* --	1-11
A	EP, A1, 0306610 (G.D. SEARLE & CO.) 15 March 1989, see the whole document --	1-11
A	EP, A2, 0214531 (MILES LABORATORIES, INC.) 18 March 1987, see the whole document --	1-11
A	EP, A2, 0028016 (GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF)) 6 May 1981, see the whole document --	1-11

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but *cited to understand the principle or theory underlying the* invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled *in the art.*

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4th February 1992	1992 -02- 06
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael G:son Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Analytical Biochemistry, Vol. 172, 1988 Mats Persson et al: "Enzyme purification by genetically attached polycysteine and polyphenylalanine affinity tails", see page 330 - page 337 see page 330, col. 2 line 11 - page 331, col. 1 line 11 --	1-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00732

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 30/11/91 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0387760	90-09-19	DE-A-	3908422	90-09-20
EP-A1- 0306610	89-03-15	CA-A-	1195627	85-10-22
		EP-A-B-	0089626	83-09-28
		JP-A-	58216697	83-12-16
		US-A-	4532207	85-07-30
		US-A-	4880911	89-11-14
EP-A2- 0214531	87-03-18	JP-A-	1168284	89-07-03
		JP-B-	3023152	91-03-28
		US-A-	4728613	88-03-01
EP-A2- 0028016	81-05-06	AT-E-	8898	84-08-15
		AU-B-	542509	85-02-21
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		JP-C-	1601560	91-02-18
		JP-B-	2023560	90-05-24
		JP-A-	57091924	82-06-08
		US-A-	4343735	82-08-10
		US-A-	4416830	83-11-22
		WO-A-	81/00571	81-03-05